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Study of Marine Biocorrosion Using AFM and Molecular Techniques

Herbert H. P. Fang *, Lichong Xu, Tong Zhang

(Centre for Environmental Engineering Research Department of Cicil Engineering, The University of Hong Kong, Hong Kong, China)

Abstract: Biofilm is ubiquitous in nature. However, corrosin caused by biofilm is still by and large overlooked. This presentation is to demonstrate the applications of several newly developed analytical techniques in chemisrty and microbiology for the study of marine biocorrosion on steel. AFM (atomic force microscopy) was applied to investigate the initial formation mechanism of biofilm and the degree of corrosion of steel in polluted seawater. DNA/RNA-related molecular techniques were used to analyze the microbial composition corrosive biofilm. Results showed that microbial corrosion began within six days, and the corroded volume in the increased as a power function of time with an index 2.83. Most of the microbes identified in the corrosive biofilm were sulfate-reducing *Desulfovibrionaceae* (46.5 %), followed by *Clostridiaceae* (29.4 %).

Key words: Atomic force microscopy, Biofilm, Corrosion, Sulfate-reducing bacteria (SRB), Steel

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1 Introduction

Due to the biochemical reactions taking place, there exists gradients of pH, dissolved oxygen, redox potential, salinity, organic and inorganic species in the biofilm[1,2]. Thus, the local conditions at the biofilm-metal interface could be substantially different from those in water. The bacterial activity at the interface could result in pitting, crevice corrosion, selective de-alloying, stress corrosion cracking or under-deposit corrosion[3]. A number of bacteria have been recognized for corrosion of metals and alloys, including iron-oxidizing[4], manganese-oxidizing[5,6], sulfur-oxidizing[7], iron-re-

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* To whom correspomdence should be addressed

ducing[8] ,and sulfate-reducing[9]bacteria. Among them ,sulfate-reducing bacteria (SRB) were the most widely recognized and studied.

In Hong Kong ,sewage of over 4. 5 million inhabitants is discharged daily into the Victoria Harbour ,mostly with only partial treatment. The sewage supplies carbon and energy for the proliferated growth of SRB ,which obtains unlimited supply of sulfate as electron acceptor in seawater. This could result in severe corrosion of marine vessels and structures ,as well as the cooling systems ,most of which in Hong Kong use seawater as the cooling medium , It has been estimated that up to 20 % of all corrosion could be due to biofilm corrosion[10]. Based on such an estimation , biocorrosion could cause Hong Kong over US $ 1 billion annually. Despite such a potential loss to the local economy , the corrsion damage caused by the discharged sewage has been overlooked. This study was thus conducted to confirm the biofilm corrosion in polluted seawater and to estimate the initial degrees of such corrosion using atomic force microscopy (AFM) and to analyze the microbial composition in the corrosive biofilm and to identify the key microorganisms responsible using DNA/ RNA-related molecular techniques.

2　Material and Methods

Mild steel coupons (10 mm ×10 mm ×1. 5 mm) were first wet polished with a series of grit SiC paper (320 ,400 ,600 ,800) ,then degreased with ethanol ,followed by further polishing with 0. 3 μm alumina particles. Seawater containing 2 200 mg/ L of sulfate was taken from the Victoria Harbour ,and sterilized by filtering through a 0. 45 μm membrane before use. The SRB seed was isolated from the marine sediment and cultured in the modified Postgate′s Medium C[11]. The pH was kept at 7. 2.

Two seawater solutions were prepared :one was added with SRB seed equivalent to 2. . 00 mg of volatile suspended solids ,and the other without the addition of SRB served as the control. Both solutions were autoclaved at 121 ℃ for 15 min ,and then purged with nitrogen to remove the residual dissolved oxygen. Resazurin was added to the solution as an anaerobic indicator. Mild steel coupons hung on nylon strings were immersed in seawater solution in air-tight glass containers. Biofilm began to grow on the coupon surfare within days. Coupons were then removed at the chosen time intervals to be examined by atomic force microscopy[12].

To prepare for AFM examination of the biofilm ,the coupons were lightly rinsed in sterile distilled water and then left to air day. In order to reveal the extent of steel biocorrosion ,the bilfilm had to removed by immersing the coupons in an ultrasonic bath at 355 W for 5 min and subsequently in a passive Clarke solution[13] containing Sb_2O_3 and $SnCl_2$ in HCI solution for 10～15 s to remove the corroded products. The exposed coupon surface was finally rinsed with sterile water , cleaned in 100 % ethanol and dried under nitrogen flow. A Nanoscope IIIA AFM (Digital Instru-

ments, USA) operating in tapping mode was used for imaging.

The DNA of the microbial community in the biofilm was first extracted, then amplified using polymerase chair reactions (PCR) and screened by denaturing gradient gel electrophoresis (DGGE). The dominant DNA in the DGGE fingerprint were then cloned and sequenced. The DNA sequences were finally compared with those of species available in the GenBank. Details of these procedures have been previously established[14].

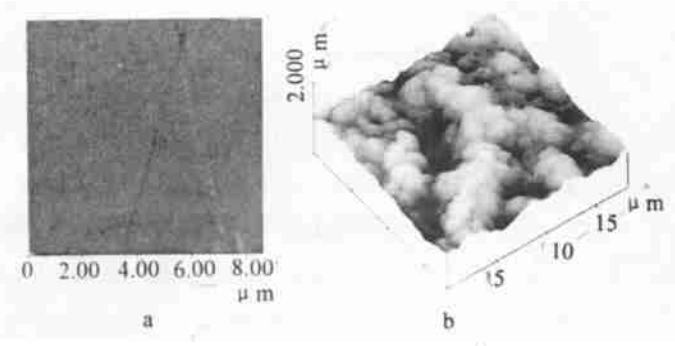

Fig. 1 Steel surface after 20 d in seawater in the absence of SRB (a), and SRB biofilm grown on steel (b)

3 Results and Discussion

Fig. 1a illustrates the surface of a control mild steel coupon after being submerged in a polluted seawater for ten days in the absence of SRB. During this period, the seawater was kept under anaerobic condition. The smooth coupon surface indicates that there was little corrosion taking place on the steel surface during this period. However, when the steel coupon was submerged in a SRB-seeded seawater, a biofilm was developed on the coupon surface within days, as illustrated in Fig. . 1b.

AFM observations of the steel surface after scrapping off the biofilm showed that pit corrosion occurred within 6 d. Fig. 2 (a～d) are the AFM images of the corroded steel surface after 10, 20, 40 and 60 d. The degree of corrosion could be quantified through section analysis of AFM images collected at various time intervals. Fig. 3 is an section analysis example conducted for a corroded steel surface after 6 d in SRB-seeded seawater, it illusrtates the depth of two pits (ca. 350～400 nm), the surface roughness, the distrbution of corroded depth, etc.

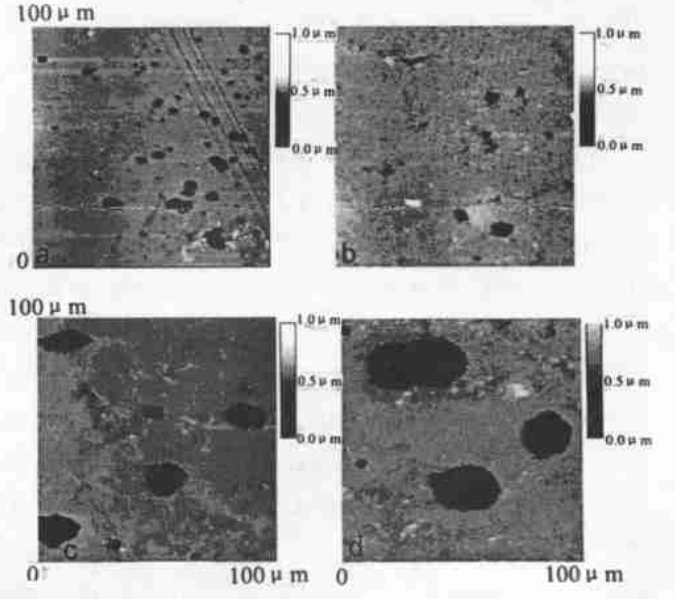

Fig. 2 Biocorrosion on steel after 10 d (a), 20 d (b), 40 d (c) and 60 d (d)

Fig. 4 illustrates the frequency of distribution of corroded pit depths at various time intervals. It shows that the degree of biocorrosion increased with time. Most of the pits were below 0.5 μm in the first 20 d, but over 2 μm after 60 d in seawater. The corroded volume increased as a power function of time with an index 2.83.

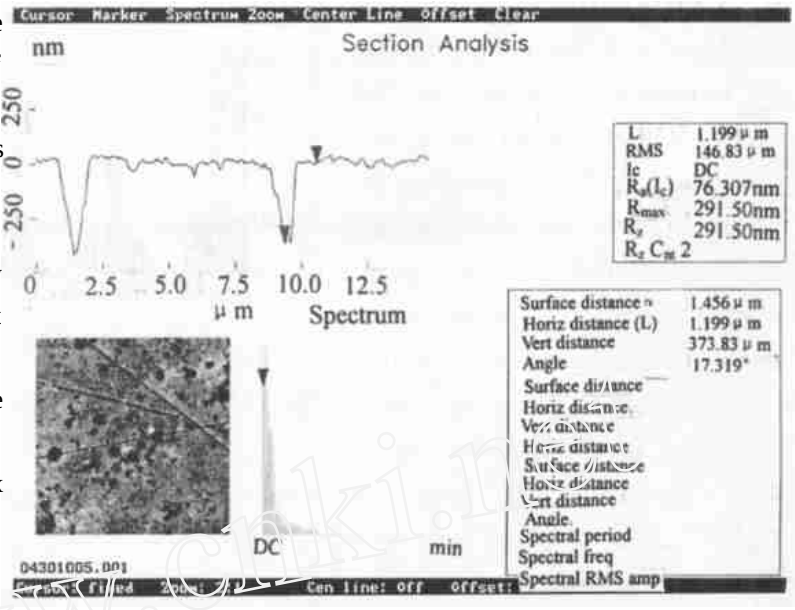

Fig. 3　Section analysis of pit corrosion of steel by AFM

Based on DNA analysis, 46.5 % of microorganisms in the biofilm were likely belonged to the *Desulfovibrionaceae* family, of which the most predominant species was *Desulfovibrio* sp. BG50 (5.0 %). The remaining were likely *Clostridiaceae* (29.4 %) and *Chlorobiaceae* (2.7 %), plus some unknown species. Details are summarized in Tab. 1.

Tab. 1　Phylogenetic affiliation of OTUs

OTU	Sequence length	Phylogenetic relationship			No. of clones	Abundance/ %
		Family	Closest species in GenBank	Similarity/ %		
90d4	613	*Desulfovibrionaceae*	*Desulfovibrio sp.* BG50	92	28	25.0
90dCB13	282	*Desulfovibrionaceae*	*Desulfovibrio caledoniensis*	93	5	4.5
90dCC9	294	*Desulfovibrionaceae*	*Desulfomicrobium baculatum*	86	4	3.6
90dCB36	379	*Desulfovibrionaceae*	*Desulfobacterium sp.* BG33	92	12	10.7
90dCC28	269	*Desulfovibrionaceae*	*Desulfobus sp.* BG25	96	3	2.7
90d10	714	*Clostridiaceae*	*Clostridium litorale*	96	17	15.2
90dCB40	349	*Clostridiaceae*	*Clostridium litorale*	96	5	4.5
90dCC18	397	*Clostridiaceae*	*Clostridium litorale*	95	2	1.8
90d11	452	*Clostridiaceae*	*Ruminococcus hydrogenotrophicus*	88	13	11.6
90dCB10	370	*Clostridiaceae*	*Ruminococcus hydrogenotrophicus*	90	7	6.3
90dCC26	410	*Chlorobiaceae*	*Chlorobium vibrioforme*	92	3	2.7
Others *					13	11.6
				Total	112	100.0

*one clone each

4 Conclusion

Results of this study confirm the formation of biofilm on the steel surface in polluted marine environment. AFM micrographs also clearly illustrate the pitting corrosion on the surface of mild steel coupons within 6 d, while there was no noticeable corrosion on the control coupons. Based on DNA analysis, most of the microorganisms in the biofilm were likely belonged to the *Desulfovibrionaceae* family (46.5 %), followed by *Clostridiaceae* (29.4 %) and *Chlorobiaceae* (2.7 %).

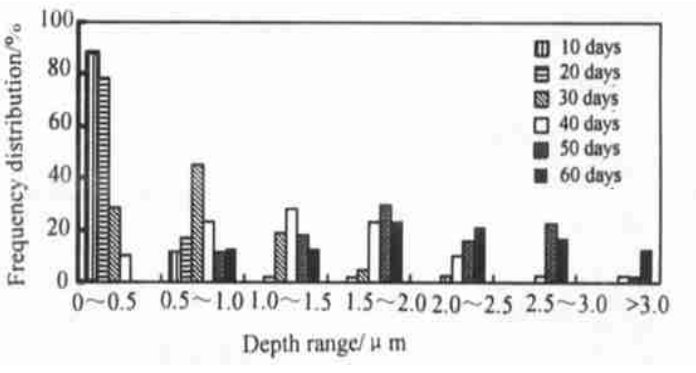

Fig. 4 Frequency distribution of pit depths over time

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利用原子力显微镜和分子技术研究海水微生物腐蚀

方汉平*, 徐立冲, 张彤
(香港大学土木工程系环境工程研究中心, 香港)

摘要: 生物膜在自然界无处不在, 但生物膜造成的腐蚀却基本上被忽视. 本文展示了几种化学和微生物学新方法在海水微生物腐蚀研究中的应用. 原子力显微镜用来揭示生物最初形成的机理和钢在受污染海水中的腐蚀程度, 16 Sr DNA/ RNA 技术则用来分析生物膜中的微生物组成. 试验结果表明, 微生物腐蚀在 6 d 内就已经开始了, 腐蚀体积与时间的 2.83 次方成正比; 腐蚀生物膜中的微生物以硫酸还原菌(脱硫弧菌科)为最多, 其次是梭状芽孢杆菌.

关键词: 原子力显微镜; 生物膜; 腐蚀; 硫酸盐还原菌; 钢